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The ability of *Anoplodinium denticulatum* Dogiel to utilize some plants

Zdolność wykorzystywania niektórych roślin przez *Anoplodinium denticulatum* Dogiel

The importance of the protozoans living in the rumen of cattle is related also to the possible participation of these organisms in the process of digesting the green fodder ingested by the ruminants.

According to Weineck 1934, all of *Ophryoscolecidae* feed on fragments of plants, some predatory species of the genus *Entodinium* excepted. On the grounds of study of the contents of endoplasmatic sacs of rumen ciliates, Liebetanz 1910 concluted that fragments of tissue serve as food for protozoans belonging to the genera *Entodinium* and *Diplodinium*. According to Dogiel 1929 a, b, Hungate 1943, and Gutierrez 1955 only the so called higher *Ophryoscolecidae* (and first of all those of the genus *Diplodinium*) absorb considerable amounts of vegetable fragments while *Entodinia*, as stated by Dogiel, feed on mixed food consisting mainly of loose grains of starch, spores of fungi, yeast, and of small vegetable fragments in an insignificant proportion only.

The correctness of the point of view of Dogiel 1929 a, b, and Gutierrez 1955 who did not admit *Entodinium* as one of the consumers of fragments of vegetable tissue in the rumen has been corroborated by the Lubinsky's 1957, 1958 data on the contents of endoplasmatic sacs of protozoans from the rumen of reindeer and from that of pakistani cattle; but first and overmost, it was corroborated by the so far obtained results of the in vitro cultures of *Infusoria* belonging to the genus *Entodinium*. Hungate 1943 and Ciemochowska-Mazik 1956 examining *Entodinium caudatum* and *Entodinium longinucleatum*, respectively, have confirmed loose grains of starch to represent the main food element of these protozoans*.

Consequently, the essential part in utilizing the green portions of plants may be played by superior *Ophryoscolecidae* only, chiefly by those from the genus *Diplodinium*.

* The results of Westphal's 1934 culture can not be taken into consideration, the species being not determined.

The cellulose decomposition has been the subject of the majority of investigations so far carried out with the aim to examine the utilization of the vegetable tissue by the rumen protozoans. However, the results of examinations were not concordant. While Trier 1926, Schultze 1924 (both after Hungate 1942), Schlottke 1936, Weinek 1934 confirmed that rumen *Infusoria* can utilize the membranes of vegetable cells, the results obtained by Margolin 1930, Westphal 1934, van der Wath 1941, and other authors were completely contrary. Unproper investigation method was doubtlessly one of the several reasons explaining for the contradictory findings.

In accordance with the opinion of Knoth 1929, Willing 1933, Westphal 1934, the investigations on the food requirements, and those on physiology of nutrition and digestion in the particular groups or species of *Infusoria* should be carried out first and foremost on the grounds of a culture in vitro; yet, a prolonged survival of the rumen protozoans beyond the host's organism has been so far achieved on very few occasions only. After Knoth 1929, Vinogradova-Fedorova 1929 (after Weineck 1934), Willing 1933, Weineck 1934, and other investigators failed, the first efficient method for laboratory culture of the rumen protozoans was prepared by Westphal 1934 (the paper by Margolin 1930 describing the in vitro culture of these organisms, which was allegedly a success, was repeatedly called in question; see Willing 1933, Westphal 1934). Mainly the infusorians from the *Entodinium* genus developed under the conditions of the Westphal's culture while *Isotrichidae* and *Diplodinia* died within 5 or 8 days. Hungate 1942, 1943, and Coleman 1958 were the first to succeed in keeping alive for several months in vivo cultures of some protozoans from the *Diplodinium* genus.

Coleman 1958 writes that also a small quantity of *Metadinium medium* (*Eudiplodinium medium*) occurred alongside several species of the *Entodinium* genus. The investigations carried out by Coleman so far delt with the methodic problems only, and among others, with that of obtaining pure cultures. On the contrary, Hungate who obtained monocultures of five species of rumen infusorians (of these, four belonged to the *Diplodinium* genus) carried out several culture observations concerning the feeding and the nutrition requirements of these organisms. He ascertained that all of the four protozoan species cultivated by him and belonging to the *Diplodinium* genus (i. e. *Eudiplodinum neglectum, E. multivesiculatum, E. maggi,* and *Anoplodinium denticulatum*) could be kept for several months on a nutrient medium the sole organic ingredient of which consisted of grass, dried and ground, or of grass and pulverized cotton cellulose.

The correctness of the assumption that the green vegetable parts might serve as food for some of superior *Ophryoscolecidae* was thus first corroborated experimentally.

Further, whilst carrying out his preliminary study on *Eudiplodinium neglectum,* Hungate noted a marked dependence of this protozoan's development on the kind of plant contained in the nutrient medium. Thus, selectiveness in respect to the vegetable material may be supposed characteristic of the other species of protozoans too.

Finally, Hungate confirmed considerable improvement in the population size of the *Eudiplodinium multivesiculatum* and *Anoplodinium denticulatum* cultures after some ground wheat was added to the nutrient medium. This would be indicative of some protozoans from the *Diplodinium* genus to utilize, besides the fragments of vegetable tissue, also some other sources of carbohydrates and protein.

The culture observations reported by Hungate can be not fully utilized owing to several essential data being omitted in the description of the experiment, and chiefly those concerning the numbers of infusorians in the cultures during particular days of culture or the conditions of collecting plants to be used as component of the protozoa nutrient medium. Further investigations are necessary in order to develop the ideas on the nutrition and food requirements of the protozoans of the *Diplodinium* genus as are suggested by the results of these observations.

Thanks be given to Prof. Dr. M. Rybicki for his suggestion as to the selection of the subject and for his kind assistance in preparing the present work.

Material and methods

The object of study was *Anoplodinium denticulatum* Dogiel 1927.

The liquid and the solid elements of the infusorians' nutrient medium were prepared before the protozoan cultures were laid. The liquid part consisted of the rumen juice of domestic cattle collected directly after slaughter; the juice was filtered through tissue-paper and steriiized for three times in Koch apparatus. Solid components of the nutrient medium were the dried up and ground leaves of the following plants: *Alopecurus pratensis* L., *Poa pratensis* L. var. *angustifolia* Sm., *Glyceria fluitans* R. Br., *Aira caespitosa* L. and *Trifolium hybridum*.

The circumstances for collection and the methods for drying up the leaves were so selected as to obtain the maximum reserve substances in the material collected. Thus, the leaves were collected at the earing period of the grasses, and — as *Trifolium* regards — one or two days before blossoming. The material was collected on a warm and sunny day, about noon; only young, intensely green leaves were selected. The leaves collected were sun-dried in a single layer. The necessary pulverization degree was obtained by grinding the dried material in a porcelain mortar and by screening through a silk gauze sack with meshes measuring approximately 75 μ. The meal thus obtained was kept in tightly sealed glass bottles protected against light and humidity.

The method for infusorian culture was in accordance with that described by Ciemochowska-Mazik 1956 except for an unimportant modification. The latter proved necessary due to the utilization of a particular method for obtaining *A. denticulatum* monocultures. A pure culture of these infusorians was obtained by catching with a micro dropper some 10 specimens of *A. denticulatum* from a mixed culture, and by putting them into the rumen juice with some *Alopecurus* and barley starch added. The method for pH regulation as utilized by Ciemochowska-Mazik turned out insufficient directly after the isolation of *A. denticulatum* from mixed cul-

tures. A sudden change in odour of the culture then occurred — probably, because of the change in the bacteria interrelation which was indicative of an excessive production of acids. 25 to 26 mg of $CaCO_3$ were added in order to stop the process of acidification of the cultures (the efficiency of this method for pH control was investigated by Willing 1933, and by other authors). $CaCO_3$ used to be added during the experiments proper so as to maintain identical conditions.

The experiments consisted in the pulverized leaves of the particular plant species being successively utilized as the solid element of the *A. denticulatum* nutrient medium.

The proportion of the particular elements in the nutrient medium was as follows: rumen juice — 10 ml., leaves of the given plant species — 50 mg., $CaCO_3$ 25 — 26 mg.

Each plant species was utilized for two kinds of culture:

1. A short-term culture lasting for 21 days (or 32 days in the case of *Aira*) covering 5 parallel cultures. At least, three of them as were notable for the number of protozoans being most similar in all of them, have been shown in tables and diagrams illustrating the results of this culture.
2. After the short-term culture was completed, one of the parallel cultures used to be left for the purpose of a long-term culture; the latter was then continued till the infusorians died out. The long-term culture was led during:

   190 days, on *Alopecurus pratensis*,
   122 days, on *Glyceria fluitans*,
   60 days, on *Poa pratensis*,
   102 days, on *Aira caespitosa*.

The *Alopecurus* culture was at the same time the control, necessary with the system of experiments being successively carried out as the case was in the work reported here.

The nutritive value of plants and their utilization by the infusoria was defined by means of the number of protozoans contained in one cc; those data were checked everyday in the short-term cultures, and every two days in the long-term ones.

The checking was carried out as follows: a part of the culture as was left behind after transferring onto a new nutrient medium was put into a refrigerator and kept there till the infusorian became immobile; next, their number in 0.1 ml was established microscopically the liquid being previously thoroughly stirred. 3 measurements were carried out for each of the cultures. The obtained mean value $\times$ 10 represented the average number of he protozoans in 1 cc. of the given culture.

Assuming that the ratio between the maximum difference in the successive measurements, on the one hand, and the average value on the other represents the measure of the standard deviation, it was established that the value of this standard deviation changed (when this particular computation method be applied) with the number of protozoans. The value of the standard deviation did not exceed 10 per cent. with more than 1000 infusorians per 1 cc.; it was approximately 15 per cent. when the infusorians numbered 500 to 1000 specimens in 1 cc., and it amounted to approximately 20 per cent. with 100

to 500 infusorians per 1 cc., while it rose even to as much as 50 per cent. when the number of the infusorians was less than 100 in 1 cc.

When new cultures were started, the infusorians used to be transferred from one of the two parallel maternal cultures onto *Alopecurus* and onto *Poa*, i.e. on plants which ensured a relatively considerable concentration of the protozoans as was shown by the preliminary experiments.

The initial level of the infusorians was approximately the same in all of the experiments with some 1600 to 2000 specimens per 1 cc.

The third or the fourth day following transfer of the infusorians onto the new vegetable species was considered as the beginning of each experiment (the only exception being *Glyceria* where it was the 7th day owing to some obstacles in reaching the proper initial level). For the question was to eliminate, by successive transplantations, the elements of the maternal nutrient medium, with which together the infusorians were transferred, prior to starting the experiments.

Experiments

1. Short-term cultures (21 days)

a. *Alopecurus* culture.

Two periods may be distinguished (Diagram I, curve 1) in analyzing the mean number of the infusorians characteristic of the particular days of the culture (Table I, Diagram I, curve 1).

The number of infusorians changes little if compared to the initial level during the first period covering the first 15 days.

The second period, beginning on the 16th day, is notable for its almost uninterrupted rise of the infusorian population reaching an average of 3655/1 cc. on the 21st day (only between the 18th and the 19th day, an insignificant decrease in the infusorian number may be observed).

The inhibition in the development of the infusorians during the first period could result from the fact of the *Alopecurus* cultures (the first experimental ones) being laid in a very short time (17 days) after the elimination of *A. denticulatum* from the mixed culture. A longer period of time seemed necessary for controlling the bacteria: infusorians ratio, disturbed by changing to the monoculture, and the process apparently ended not sooner than in the *Alopecurus* culture.

It also ought to be emphasized that the starting the *Alopecurus* culture implied the change in the character of food (nutrient medium of rumen juice and grass was substituted for that of rumen juice, grass and starch). Absence of starch probably induced changes in the composition of the bacterial flora and consequently, in that of the medium of the culture which, in turn, could induce a temporary inhibition in the infusorians development (from 1 to 15 days).

The data in Table I make it obvious that the development was similar in all of the three parallel *Alopecurus* cultures. More significant differences

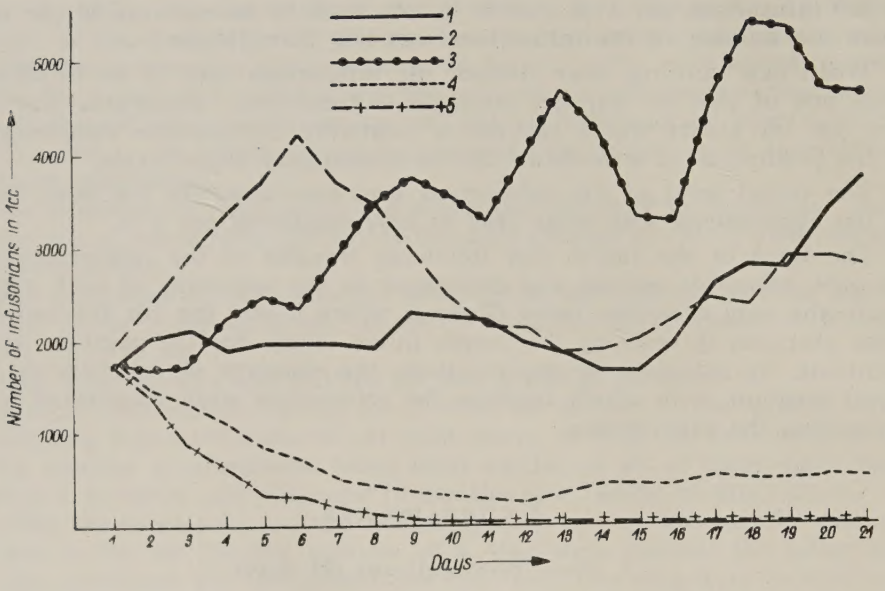

Diagram I. Average numbers of infusorians in cultures on various plants
1. *Alopecurus pratensis* L.; 2. *Poa pratensis* L.; 3. *Glyceria fluitans* R. Br.; 4. *Aira caespitosa* L., 5: *Trifolium hybridum*

occurred during the periods between the 1st and 6th day, and between the 16th and 21st day. At the same time, the character of the curse was concordant in all of the cultures, with culture No. 3 exhibiting the lowest number of infusorians almost over the whole period of culture.

b. *Poa* culture.

During the initial period of the *Poa* culture (from the 1st to the 6th day), a rapid increase in population size of the infusorians may be observed which stops when the average of 4166 specimens to 1 cc. is reached (Table II, Diagram I, curve 2). This is the maximum average number of infusorians obtained in a short-term culture on *Poa pratensis*. A gradual fall occurs between the 6th and 13th day, the infusorians averaging then 1815 specimens to 1 cc. (which was the lowest average number registered during the 21-day period). A slow increase in number of infusorians sets in on the 14th day, lasting till the 19th (except for the short period between the 17th and 18th day of culture when there is an insignificant fall) and ends with an average level of 2810 specimens to 1 cc. This number insignificantly diminishes during the last two days of cultivating.

During the whole experimental period, the three parallel cultures behaved very similarly.

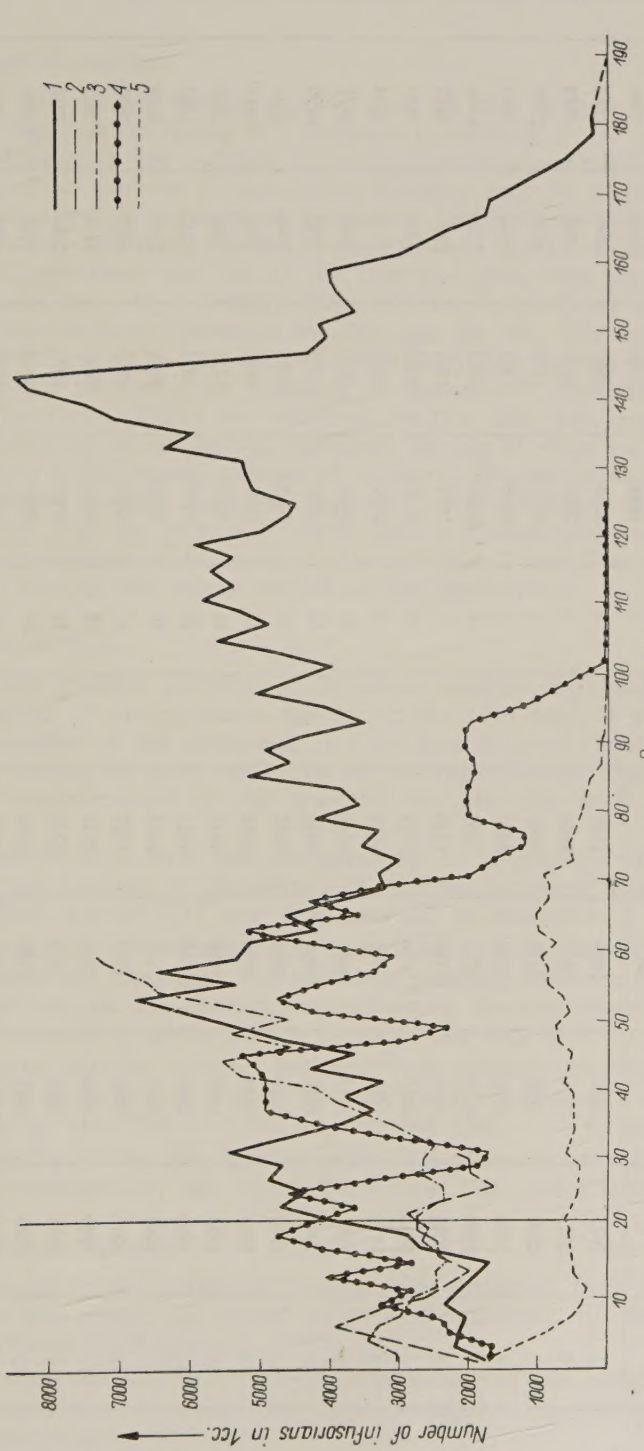

Diagram II. Culture of *A. denticulatum* on various plants

1. *Alopecurus pratensis* L.; 2. *Poa pratensis* L. — I; 3. *Poa pratensis* L. — II; 4. *Glyceria fluitans R. Br.*, 5. *Aira caespitosa* L.

Table I

Cultivation of A. denticulatum on Alopecurus on 27.XII.1958 - 16.I.1959

Days of culture	Number of protozoans in 1 cc. in cultures number:			Average
	1	2	3	
1	1673	1736	1654	1687
2	1733	2306	1973	2004
3	2320	2156	1773	2083
4	1740	2130	1703	1857
5	1986	2126	1700	1937
6	2063	2016	1736	1938
7	2066	2033	1696	1931
8	1976	1950	1703	1876
9	2350	2313	2086	2249
10	2340	2216	2090	2215
11	2310	2150	2086	2182
12	1900	1970	1956	1942
13	1853	1900	1696	1816
14	1653	1666	1516	1611
15	1663	1670	1516	1616
16	2016	2066	1700	1927
17	2790	2650	2096	2512
18	2933	2920	2353	2735
19	2920	2863	2346	2709
20	3716	3343	2750	3269
21	3926	3860	3180	3655

Table II

Cultivation of A. denticulatum on Poa on 20.I. - 9.II.1959

Days of culture	Number of protozoans in 1 cc. in culture number:			Average
	1	2	3	
1	1933	2006	1833	1924
2	2153	2620	2340	2371
3	2810	3003	2826	2879
4	3033	3776	3086	3298
5	3476	3993	3520	3663
6	3950	4613	3936	4166
7	3456	4103	3496	3685
8	3376	3610	3370	3452
9	2726	2920	3110	2918
10	2446	2226	2640	2437
11	2040	1963	2293	2098
12	2053	1940	2340	2111
13	1920	1676	1850	1815
14	1906	1733	1960	1866
15	1960	1823	2136	1973
16	2100	2096	2380	2192
17	2250	2340	2606	2398
18	2246	2263	2426	2311
19	2700	2670	3060	2810
20	2870	2810	2736	2805
21	2766	2720	2686	2724

c. *Glyceria* culture.

A steady increase tendency is notable for this culture during almost the whole 21-day period (Table III, Diagram I, curve 3).

The changes in the number of protozoans are spasmodic and occur rather regularly. Four periods of population increase may be distinguished in analyzing the average numbers of *Infusoria*; the two first ones (from the 2nd to the 5th day and from the 6th to the 9th day) last for three days, while the two next ones (from the 11th to the 13th day, and from the 16th to the 18th day) last for two days; besides there are four periods of decrease covering 1 to 3 days intervals (between the 5th and the 6th, 9th and 11th, 13th and 16th, and between the 18th and the 21st day). During the successive periods of population increases the numbers of infusorians reach higher and higher levels. The figure 5333/1 cc. recorded on the 18th day, was the maximum average number of protozoans obtained during 21 days on *Glyceria;* at the same time it is the highest number of *Infusoria* obtained in short-term cultures.

The three parallel cultures behaved very similarly during the whole experiment. On the contrary, there was a considerable difference in the respective numbers of *Infusoria* in the cultures 2 and 3 as compared to the culture 1 during the whole period of the experiment.

d. *Aira* culture.

A steady gradual decrease of *Infusoria* number was observed during the initial period of culture (from the 1st to the 12th day); it continued till the average number of 248 specimens in 1 cc. was attained (Table IV, Diagram I, curve 4). During the next two days, the average number of *Infusoria* reached 431/1 cc. and remained at this level till the 21st day with insignificant variations.

The period of culture was extended up to 32 days in order to ascertain whether the increase of the *Infusoria* number observed after the minimum was attained (on the 12th day) was constant in character.

No significant differences in the protozoans number were observed between the 21st and the 32nd day, the variation ranging between 516 and 313 specimens in 1 cc. on the average; consequently, the variations were similar to those characteristic of the period between the 14th and 21st day.

The three parallel cultures behaved very similarly during the whole period of the experiment, except for culture 3 exhibiting a more significant deviations on the 12th — 14th and on the 15th — 17th days.

The results of the *Aira* culture show this plant to be utilized by *A. denticulatum* considerably less than *Alopecurus, Poa,* and *Glyceria.*

e. *Trifolium* culture.

A decrease in the *Infusoria* number was observed from the first day of culture on this plant; this lasted, with short intervals, till the whole culture perished (Table V, Diagram I, curve 5).

There were marked differences in behaviour of the parallel cultures during the experiment on *Trifolium;* they consisted in the protozoans dying out at different rates in the various cultures.

Table III

Cultivation of A. denticulatum on Glyceria on 25.III.-14.IV.1959.

Days of culture	Number of protozoans in 1 cc. in culture number:			Average
	1	2	3	
1	1773	1723	1710	1735
2	1740	1616	1670	1675
3	1640	1670	1813	1707
4	2476	1730	2413	2206
5	2663	2290	2363	2438
6	2600	2153	2183	2312
7	3596	2390	2606	2864
8	4170	2883	3210	3421
9	4806	3273	2996	3691
10	4530	2975	2976	3493
11	4180	2833	2676	3229
12	5046	3403	3250	3899
13	5676	4076	4116	4622
14	5430	3533	3553	4172
15	4213	2790	2826	3276
16	4156	2726	2766	3216
17	5336	4096	4160	4530
18	6076	4860	5063	5333
19	6096	4740	4983	5272
20	5316	4183	4283	4594
21	5360	4116	4233	4569

Table IV

Cultivation of A. denticulatum on Aira on 14.II.-17.III.1959

Days of culture	Number of protozoans in 1 cc. in cultures number:			Average
	1	2	3	
1	1820	1680	1856	1785
2	1440	1416	1546	1467
3	1316	1243	1403	1320
4	1043	1080	1206	1109
5	796	793	920	836
6	676	670	783	709
7	450	496	550	498
8	383	420	436	413
9	343	353	383	359
10	286	313	330	309
11	263	283	280	275
12	246	233	266	248
13	270	290	426	328
14	446	406	443	431
15	446	400	463	436
16	383	373	526	427
17	480	473	540	497
18	456	456	530	480
19	436	453	513	467
20	496	473	566	511
21	480	483	556	506
22	416	430	480	442
23	413	400	453	422
24	413	383	440	412
25	360	336	410	368
26	353	340	390	361
27	336	310	376	340
28	336	303	363	334
29	286	293	360	313
30	386	390	503	426
31	496	476	576	516
32	403	380	466	416

T a b l e V

Cultivation of A. denticulatum on Trifolium on 18.IV. - 8.V.1959

Days of culture	Number of protozoans in 1 cc. in cultures number:					Average
	1	2	3	4	5	
1	1696	1666	1640	1716	1730	1689
2	1340	1413	1380	1323	1356	1362
3	723	950	853	906	906	867
4	313	906	713	350	833	623
5	136	463	393	233	486	342
6	90	453	376	160	446	305
7	103	300	256	70	263	198
8	56	210	166	40	180	130
9	33	120	66	23	86	65
10	6	76	46	3	53	37
11	10	93	23	3	26	31
12	6	50	6	3	3	13
13	0	60	3	3	3	-
14	3	26	0	0	3	-
15	0	36	0	0	3	-
16	0	23	0	3	0	-
17	0	16	0	0	0	-
18	3	13	0	3	0	-
19	0	20	0	0	0	-
20	0	6	0	0	0	-
21	0	13	0	0	0	-

T a b l e VI

Plant	Proportion of the different size fragments		
	less than 20 µ	20 to 80 µ	80 to 200 µ
Alopecurus	88	9.0	3.0
Poa	85	12.5	2.5
Glyceria	92	7.5	0.5
Trifolium	88	11.0	1.0
Aira	89	7.0	4.0

This rate was the highest in the cultures 4 and 1 : the infusorians numbered here less than 10/1 cc. after 9 days which, practically, was equal to extinction. Less than 10 specimens in 1 cc. were recorded on the 12th day in the cultures 5 and 3, while the protozoans survived for as long as 21 days in culture 2 where their number varied between 10 and 20 specimens in 1 cc. from the 17th day onwards. No protozoans were confirmed in culture 2 during the following three days.

The extinction of these cultures would be indicative of *Trifolium* being not a proper source of nutrient substances for *A. denticulatum*. On the other hand, the extinction of infusorians might be supposed to occur much earlier than it did in fact should *Trifolium* represent no value at all as a nutrient.

The infusorians may therefore be supposed to utilize *Trifolium* to a very insignificant extent, and the extinction of the cultures may be ascribed to some new factors, injurious to the protozoans development, which factors showed up under the conditions of culture.

Comparing the results of short-term cultures

The number of infusorians in culture was established to depend closely on the plant species in the nutrient medium.

The highest number of protozoans, namely an average level of 5333 specimens per 1 cc. was attained in a *Glyceria fluitans* culture. A slightly inferior level was obtained with *Alopecurus pratensis*, and with *Poa pratensis*, (3655/1 cc. and 4166/1 cc., respectively).

In the *Aira caespitosa* culture, the infusorians first decreased in number to remain then steadily within the limits of 516 to 313 specimens per 1 cc. on the average.

The *Trifolium hybridum* culture perished after 21 days (Diagram I).

The changes in number of protozoans (as were observed during the course of the culture) were gradual in character on the *Alopecurus, Poa*, and *Aira* cultures, while they were spasmodic in the *Glyceria* culture.

2. Long-term cultures

a. *Alopecurus* culture.

The first three weeks of this culture were reported above when speaking about the short-term cultures (Table I, Diagram I, curve 1). The number of protozoans during the following 169 days is represented by the curve 1 in Diagram II.

Relatively regular changes in the *Infusoria* numbers were established during the period from the 22nd to the 93rd day of culture. Three principal periods of gradual increase may be distinguished, if insignificant variations be neglected (the first one — the continuation of the increase as started on the 16th day and lasted untill the 31st day; the second — between the 41st — 53rd and the third between the 73rd — 85th day), and three corresponding periods of gradual decrease (between the 31st and the 41st day, the 53rd and the 73rd day, and between the 85th and the 93rd day, respectively). As a result of the successive periods of increase, the number of protozoans per 1 cc.

attains the following magnitudes: 5443, 6833 and 5196; following the periods of decrease it successively falls to 3186, 2983 and 3460 specimens per 1 cc.

The curve exhibits a marked growth tendency between the 93rd and the 143rd day of culture. The maxima of the curve, observed during this period, climb always higher and higher. At first (between the 93rd and 125th day) the increase is interrupted by decreases of short duration; this increase becomes almost uninterrupted from the 126th day on, ofter a more considerable fall (between the 120th and 125th day).

The number of 8540 protozoans per one cc. recorded on the 143rd day was the highest obtained in the *Alopecurus* culture. It was also the highest number of infusorians attained during the whole work reported here.

A violent fall set in on the 144th day of culture, and the number of *Infusoria* dropped to 4300/1 cc. three days later. A new fall occurred after 12 days, and lasted uninterruptedly for 31 days. The culture was concluded after 190 days with approximately 10 infusorians per 1 cc.

A marked change in the odour of the culture was established during the last month; this would be indicative of a change in the direction of the fermentation processes.

b) *Poa* culture.

The first 21 days of this culture were reported above when speaking of the short-term cultures (Table II, Diagram I, curve 2).

Further results are shown by the curve 2 in Diagram II.

Following the initial fall between the 22nd and the 26th day (1576/1 cc.), the number of *Infusoria* increases to 2403/1 cc. (on the 32nd day) to reach then a similar level as was recorded on the 22nd day (2726/1 cc.). The culture perished on the 32nd day. An additional monoculture was started on *Poa*. The course of the latter is depicted by the curve 3, Diagram II. Following an initial increase, the number of the protozoans gradually dropped till 2210/1 cc. was attained (on the 16th day). Similar to this was the next major fall of the curve as occurred on the 24th day following an insignificant increase. There were no marked changes in the infusorian population size between the 24th and 32nd day. The population began increasing on the 33rd day; this continued, with a short interval, up to the 60th day when 7463 infusorians per 1 cc. were registered. This figure considerably exceeded the two previous maxima of the curve. After 60 days the culture was discontinued due to the *Poa* stock being exhausted.

c. *Glyceria* culture.

The course of the first three weeks of this culture was reported above in speaking of the short-term cultures (Table III, Diagram I, curve 3).

The curve 4 in Diagram II depicts further results of the culture.

During the period between the 22nd and the 65th day, changes in the size of the protozoan population occur spasmodically at rather regular and short intervals, with a considerable amplitude.

The curve as depicts these changes shows four maxima (on the 25th, 45th, 53rd, and 63rd day), nearly at the same level, and four minima.

There occurred a distinct change in the character of the culture after the

64th day. Following an insignificant increase, the number of *Infusoria* dropped to 1176 specimens per 1 cc. (on the 75th day), i.e. the fall is much more marked than in all of the previous turns of the curve. As a result of the successive increase, the population size reaches as little as 2040 specimens per 1 cc. (on the 81st day); this level remains unchanged, with insignifficant variations, for the next 12 days. Then, a new decrease follows.

The culture was ended after 125 days, with less than 10 protozoans per 1 cc. The extinction of the protozoans was concomitant with a change in the odour of the culture, similar to that observed in the long-term *Alopecurus* culture during the end period of the latter.

d. *Aira* culture.

The course of *Infusoria* development in this culture during the first 32 days was reported above when speaking of the short-term culture (Table IV, Diagram I, curve 4). Further results are illustrated by the curve 5 in Diagram II.

There were no significant variations in size of the protozoan population during the period from the 32nd to the 45th day. The curve shows a marked tendency towards increasing during the next 20 days. The successive maxima climb always higher and higher during this period. 1000 infusorians per 1 cc., recorded on the 65th day, was the highest number obtained on the *Aira* culture.

The population size begins to drop on the 66th day and, after two short intervals, goes on (from the 75th day on) falling uninterruptedly to less than 10 specimens per 1 cc. (on the 103rd day).

A noticeable change in odour of the culture was recorded during the steady period of the decrease in size of the infusorian population; the odour resembled that as was noticed when the long-term protozoan cultures perished as were grown on *Alopecurus* and on *Glyceria*.

Comparing the results of the long-term cultures

The highest number of infusorians, 8540/1 cc., was obtained in the *Alopecurus* culture (Diagram II, curve 1); lower was that obtained on *Poa* with its maximum = 7463/1 cc. (Diagram II, curve 3), and on *Glyceria* with its maximum = 5260/1 cc. (Diagram II, curve 4); the lowest number of infusorians was obtained on the *Aira* culture with its maximum = 1000/1 cc. (Diagram II, curve 5).

The infusorian cultures on *Alopecurus*, *Poa*, and *Aira* exhibited common features to some extent (Diagram II, curves 1, 3 and 5). After a period of a relative stabilization in changes of the amplitude (between the 19th and the 93rd day in *Alopecurus*, between the 1st and the 32nd day in *Poa*, and between the 14th and 45th day in *Aira*), followed a longer period of gradual increase of the protozoan population (lasting for 93 — 143 days in *Alopecurus*, 32 — 60 in *Poa*, and 45 — 65 in *Aira*); at the end of this period, the highest number of infusorians on the respective plant culture was obtained.

In the case of the *Glyceria* culture, however, the relatively long period of gradual increase in size of the infusorian population (at the end of which period the figure 4860/1 cc. was attained, i.e. a figure very similar to the maximum of 5260/1 cc. attained on the 63rd day) fell onto the beginning of the culture (between the 1st and the 18th day, see Table III, Diagram II, curve 4). Considerable regularity was characteristic of further development of the culture, up to the period preceding the extinction of the infusorians (i.e. up to the 65th day).

All of the long-term cultures ended in extinction of the infusorians (the *Poa* culture excepted; it was discontinued after 60 days); the infusorians perished after 190 days on the *Alopecurus* culture, after 122 days on the *Glyceria* one, and after 102 days on the *Aira* culture.

In all of these instances a distinct change in odour of the culture was established during the final period of culture.

Discussion

The ground leaves of different plants utilized in the experiments reported here were not the only source of organic substances available to the infusorians. The liquid component of the nutrient medium, the rumen juice, doubtlessly contained some amount of these substances too, in the shape of bacterial protein and in that of various products of bacterial fermentation. Taking into account, however, that distinct differences occurred in size of the *A. denticulatum* population (see Diagram I and II) in cultures laid on different plants, but equal in their contents of rumen juice, the limited components of the latter may be assumed to have had no substantial significance in this protozoa nutrition. The rumen juice seems to be infusorians' source of such elements as mineral salts, vitamines etc. both under natural conditions and in cultures in vitro.

Besides the *Infusoria*, also bacteria developed in the culture medium, and first of all, those as were transferred from the rumen together with the infusorians although the methods applied did not eliminate the introduction of other bacteria from outside. If follows from the observations carried out by several authors — Liebetanz 1910, Schouten 1928 and Lepsi 1927 (both after Ferber 1928), Ferber 1928, Westphal 1934, van der Wath 1941, Hungate 1943, Gutierrez 1955, Lubinsky 1957 — 1958, and others — that infusorians absorb bacteria. Consequently, *Anoplodinium denticulatum* too could utilize bacterial protein. Inspection of the contents of the endoplasmatic sacs (which was done regularly during the whole period of culture) revealed considerable amounts of vegetable fragments in most of the *A. denticulatum* specimens; protozoans absorbing tissue fragments were observed on several occasions. Even if the infusorian examined should really feed on bacteria, this ought to be considered not sufficient to meet all of its feeding requirements.

Thus, the results of the experiments enable us to draw the conclusion that *Anoplodinium denticulatum* in culture utilized — at least in part — nutritive substances composing the cells of the green tissue of plants. At the same time, the considerable number of infusorians obtained in cultures

on certain plants (8540/1 cc. in the *Alopecurus* culture, Diagram II, curve 1) seems to eliminate the probability of the protozoans feeding exclusively on the contents of the vegetable cells, as were devoid of their cellular membrane while ground. Taking into account the grinding methods applied, the number of the destroyed cells was probably not significant; microscopic observations revealed the presence of a considerable amount of larger fragments of plant tissues.

In view of the contradictory results of investigations on the occurrence of the particular digestive enzymes in the rumen infusorians, it is difficult, for the time being, to give any particular data on the mechanism for utilizing, by these organisms, the organical substances contained inside the vegetable cell. Should the opinion — supported by Schuberg 1888, Braune 1914, Schultze 1924, Trier 1926 (all four after Hungate 1942), Liebetanz 1910, Weineck 1934, Scholtze 1936, Hungate 1942 — 43, and others — on the rumen infusorians having cellulolitic enzymes prove correct, then their access to the inside of the vegetable cell would be difficult for them. Should, however, the rumen protozoans have no such enzymes — accordingly to the opinion expressed by Mangold 1929 (after van der Wath 1941), Margolin 1930, Westphal 1934, and van der Wath 1941 — then the view of Mangold 1929 and wan der Wath 1941 ought to be accepted, namely that the enzymes of bacteria ingested by the infusorian together with food participate at the decomposition of the walls of the vegetable cells swallowed by the protozoan.

Van der Wath considers that the participation of bacteria at the digestive processes take place in the endoplasmatic sac of the rumen protozoans is not limited to the decomposition of the cellulose but extends also to the digestion of starch. Ferber 1928 supposes that the fat stuffs absorbed by the infusorians are decomposed in the same manner. On the other hand, Eadie 1956 in discussing the mechanism of the utilization of laminarin (a hydrocarbon occurring in *Laminaria* sp.) by some of the holotrichous ciliates suggests alongside other possibilities also that of the vegetable enzymes participation at this process. Van der Wath 1941 mentions the vegetable enzymes as an important factor in food decomposition in the cattle rumen. Consequently, the vegetable enzymes may also constitude a substantial link in the process of the utilization of the organical substances of the vegetable cell by *Anoplodinium denticulatum*.

It results from the in vitro culture that the utilization degree of various plants by *A. denticulatum* is not the same. This corroborates the preliminary observation carried out by Hungate 1942 on *Eudiplodinium neglectum*: according to this author's observations, the rumen protozoans were selective in relation to green fodder (it would be interesting to know that, in the case of *Eudiplodinium neglectum*, a plant from the *Papilionaceae* family — *Medicago arabica* turned out the less appropriate nutrient).

In order to ascertain whether the differences in the infusorian population size on various plants were not related to the dimensions of the vegetable fragments, necessary measuring were carried out; the results are compared in Table VI.

It results from the data in Table VI that the proportion of vegetable fragments smaller than 20 μ was similar in all of the cases. Especially these

tissue fragments seemed to play the decisive part in the protozoans nutrition. Although the particular plants exhibited some variations in the contents of fragments belonging to the two remaining size groups (especially to the third one — 80 to 200 μ), still they were not congruent with the results of the culture. For, the highest amount of vegetable fragments within the 80 to 200 μ size range was contained in *Aira* with its low level of protozoans, on the one hand, and in *Alopecurus* in which the protozoans developed much better, on the other. Also *Glyceria* and *Trifolium* are notable for containing similar amounts of fragments within the 80 to 200 μ range; yet, the results obtained in cultures grown on these two plants were quite different. Consequently, these results are indicative of the size of the vegetable fragments being not the cause of the differences observed in the *A. denticulatum* development on the particular plants. Hence, the selectivity might be supposed to depend on the chemical composition of the plants and, probably, on the composition of amino-acids contained in vegetable protein first and foremost. A closer examination of this problem is made impossible due to reliable and comparable data on the chemical composition of plants utilized in cultures lacking in the literature available.

On the other hand, the development degree of *A. denticulatum* (depending on the vegetable component of the nutrient medium) might also be one of the factors telling on the size of population of the protozoans as they might ingest also bacteria under the conditions of culture. The significance of this factor can be hardly defined at present due to the lacking data on the bacterial flora occurrence in cultures grown on the particular plants.

Alongside distinct differences occurring in the development of *A. denticulatum*, also some regular features were confirmed to be common to various plants. In the *Alopecurus*, *Poa*, and *Aira* long-term cultures during a latter period of culture, a prolonged, gradual increase in population size of the infusorians was noted which ended in reaching the highest protozoan population with that particular plant (see Diagram II, curve 1, 3, 5). In all of the cases, this process set in nearly at the same time, namely at the end of March and the beginning of April. This is shown in Diagram III illustrating the course of *A. denticulatum* culture on various plants arranged according to real calender succession. The phenomenon mentioned above enables us to suppose some additional factors affecting the infusorians and rising the intensity of their propagation during the early spring. The development process of the protozoans on *Glyceria* is another corroboration of this supposition. In a long-term *Glyceria* culture laid on March 25th, the infusorian population rose to 4860/1 cc., i .e., to a level very similar to the maximum (5260/1 cc.) as early as on the 18th day of culture (Diagram II, curve 4).

Further analysis of Diagram III shows that the temporary rise in the infusorian population, as set in nearly at the same time in all of the cultures, was somewhat different in character on each plant. First of all there was a difference in its duration; in the *Aira* (Diagram III, curve 5) and *Glyceria* (Diagram III, curve 4) cultures, the highest or nearly the highest number of population fell on April, while it fell as late as on May 18th in the *Alopecurus* culture (Diagram III, curve 1); as to the *Poa* culture, it can not be taken into consideration as it is not known whether the number of infusorians recorded on the 60th day represented the actual maximum for this plant.

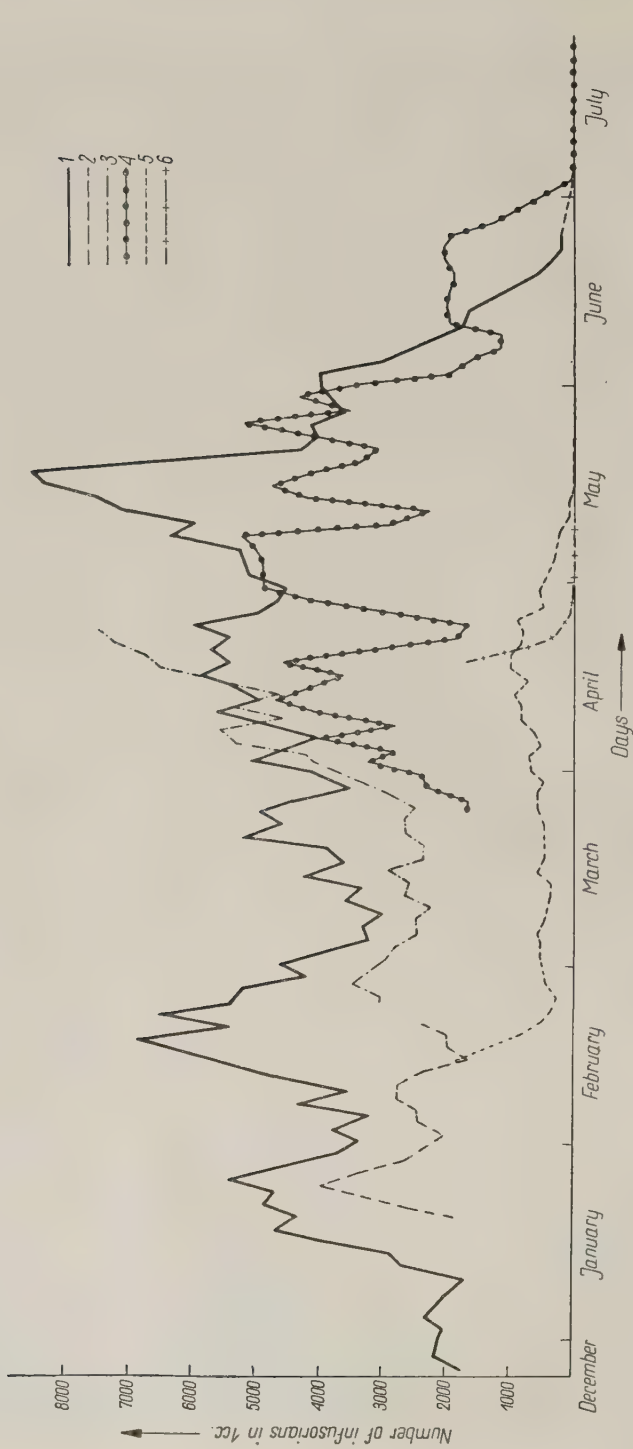

Diagram III. Culture of *A. denticulatum* on various plants

1. *Alopecurus pratensis* L.; 2. *Poa pratensis* L. — I; 3. *Poa pratensis* L. — II; 4. *Glyceria fluitans* R. Br.; 5. *Aira caespitosa* L.; 6. *Trifolium hybridum*.

Further, the relative value of the infusorian population size increment, attained as the result of the temporary population growth, varied on the particular plants too. The growth increment was of the order of 100 per cent. in the *Poa*, and *Aira* cultures as it resulted from comparison of the protozoans level on the 60th resp. 65th day of culture, on the one hand, to the number attained on the 4th resp. 31st day when the maximum was reached during the preceding period. In the *Alopecurus* cultures, the increment was considerably less significant (compare the population size on the 143rd day to that on the 53rd day, Diagram II).

Several authors investigated the problem of the temporary changes in the population size of *Protozoa* in their natural medium, i.e. in the contents of the cattle rumen.

Van der W a t h 1941, confirmed in his experiments on the South African rams that these changes were related to the composition of food ingested by the host. During the period from October to January when the contents of reserve substances (especially those of protein) reached their highest point in the grasses under the South African conditions, the population size of the infusorians in the rumen of the rams increased by several times (from 2 times to more then 4 times) as compared to July when the fibrous elements prevailed in grass.

Under the conditions of the temperate climate, a significant improvement of the nutritive value in the cattle feeding occurs during the spring period due to the animals going over to the fresh green fodder. Consequently, on the grounds of the van der W a t h's 1941 findings, the protozoan population in cattle may be supposed to begin increasing in size during the spring period under our conditions, although no necessary studies were carried out so far.

There is still another factor as sets in the spring and brings about changes in the concentration of *Infusoria* in the cattle rumen: spring is the season of breeding, and pregnancy and lactation period coincide with an approximately twofold increase in the protozoan population as confirmed by F e r b e r 1928, and M o w r y et B e c k e r 1930 (in goats).

It may be admitted that factors bringing about the springtime increase in the infusorian populations in the cattle rumen during many years resulted in a certain regular recurrence in the development of the protozoans even in cultures in vitro.

The chemical composition of the particular plants (on which the possible development of the infusorians depends to a considerable extent) may account for the differences in the growth processes as were observed in infusorians on various plants.

On the other hand, the composition of the rumen juice may be supposed subject to some modifications as a result of cattle passing over from the winter food to the spring one. It is possible that the increase in the protozoan population size, as was recorded in the culture during the early spring period, was a reaction induced by the change in the composition of the liquid part of the nutrient medium for the employed rumen juice used to be changed (approximately) every three weeks. In view of such a possibility, the relatively significant increase in the infusorian population size in the *Poa* and *Aira* cultures occurring in mid April would be indicative of the rumen juice

being — for the infusorians in both of these cases — a source of some substances necessary for the development which substances either did not occur in the solid component of the nutrient medium (i.e. in the leaves of *Poa* and *Aira*) or occurred in limited amount. Similarly, in the case of *Alopecurus*, first of all the ground leaves should be regarded as the source of all the components necessary for normal development, while the part of the rumen juice was quite insignificant.

To date, no sufficient data are available to decide which of these two factors was the real cause of the simultaneous increase of the number of infusorians on all of the plants during the spring period. In future, the above experiment should be repeated utilizing the same rumen juice during the whole period of culture in order to resolve this problem.

Although the effect of some outside factors is very likely to act under the conditions of culture during the spring period, yet the essential results of the experiments carried out are still important. The differences in the infusorians number on the various plants as were confirmed previously (Diagram III) have remained also in this period disregarding some similarity in the character of development of the particular cultures.

According to D o g i e l 1929, all of the representatives of the *Diplodinium* genus occurring in the rumen of cattle number some 8000 specimens per 1 cc. The number of 8540/1 cc. obtained in the *Alopecurus* culture seems rather large if compared to the figure of D o g i e l. The considerable number of infusorians obtained during the experiments reported here, and the fact of some cultures being a success during a long period (the protozoan population on the *Alopecurus* culture began decreasing after 5 months) show that the methods of culture applied enabled to create conditions proper to meet the requirements of the organism examined. Therefore, the final extinction of the long-term cultures on *Alopecurus, Glyceria* and *Aira* ought to be considered a fortuitous phenomenon induced by some of the factors having caused a modification in the conditions of culture.

The symptoms attendant the extinction of the protozoans in these cultures (the change in odour) and the almost identical period of extinction of all of the cultures (Diagram III) are indicative of the extinction cause being probably the same in all the cases. The more early extinction of the *Aira* culture should be related to the smaller population of the infusorians which fact is known to make them much more sensitive to noxious factors.

The analysis of the conditions of culture permits to suggest several possible reasons for the extinction of the protozoans.

The change in odour during the final period of experiments could be indicative of the culture medium being dominated by another sort of bacteria which changed the course of the fermentation processes, harmful to the infusorians.

In order to restitute the normal bacterial relations, the unsterilized rumen juice was substituted for the sterilized one (freed of infusorians by filtering). However, this failed to improve the culture conditions. The inhibition of the course of the fermentation processes might be also induced by the changes in the composition of the vegetable nutrient medium due to its being stored too long. Yet, the utilization of a new portion of plants (collected and dried under the previously described conditions) did not stop the dying-out process

of infusorians. Consequently, the extinction of the colony should be related to some other factors. A successive portion of the rumen juice obtained in the slaugher-house could well contain some substances harmful to the development of protozoans (e. g. the juice might be collected from an ill animal or from one treated with antibiotics).

Dogiel 1929 maintains that the infusorian population in the cattle rumen is somewhat less in the summer than in the winter period. Most of the clones of *Anoplodinium denticulatum* cultivated by Kill 1940 in goats perished in May and June. This would be indicative of the summer period being less favourable to the infusorian development; perhaps, it ought to be related to the ripening of grasses and the resulting changes in the rumen juice composition. In the case of the culture reported here, the above factor could have accelerated the extinction of the protozoans in long-term cultures on *Alopecurus* and *Glyceria*. The data available do not permit to draw any definitive conclusions as to the causes of the failure of the culture; the problem of the effect of some of the factors reported here (the season, and others) exert on the infusorian development can be solved solely by means of further investigations.

Some objections as to the results of the *Trifolium* culture will ensue from the analysis of Diagram III; thus, the definitive extinction of the infusorians on *Trifolium* might well have been induced by the effect of the same factors as have brought about the protozoans dying out on *Aira* and, following a longer period, on *Alopecurus* and on *Glyceria* as well. Thus, it is not impossible that, under unchanged conditions, *Trifolium* could ensure the development of a small number of infusorians. The duration of the process of the protozoa extinction on this plant (Table V) is a fact that would support the above hypothesis.

The infusorians' number obtained in the experiments reported here (8540/1 cc. on *Alopecurus*) exceeds the population size of *Anoplodinium denticulatum* characteristic of the Hungate's cultures — 100 to 300 specimens per 1 cc. Also the duration of cultures was longer on some plants — 190 days on *Alopecurus* — exceeded that of *A. denticulatum* as reported by Hungate (2 months). These facts are indicative of the organic base of culture utilized (i.e., the rumen juice) giving much better conditions for *A. denticulatum* development than the solution of inorganic salts utilized by Hungate. The insignificant population sizes of protozoans in the Hungate's cultures might be also the result of inapropriate selection of plants utilized as nutrient medium for the infusorians, for the experiments reported here have shown *Anoplodinium denticulatum* to be selective in respect to the green food.

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STRESZCZENIE

Anoplodinium denticulatum hodowano w kulturach in vitro w warunkach anaerobowych stosując jako podłoże sok żwacza, a jako pokarm suche liście czterech gatunków traw i koniczyny czerwonej. Miarą wyzyskania poszczególnych roślin była ilość wymoczków w 1 cm^3 soku kultury, kontrolowana co 24 godziny w ciągu całego okresu hodowli.

Wyniki eksperymentalne wskazują, że *A. denticulatum* odżywia się roślinami, czego dowodem jest wypełnienie ich woreczków endoplazmatycznych fragmentami roślin oraz ilość wymoczków w 1 cm^3 w zależności od rośliny. Największą liczbę wymoczków (średnio 5 333 egzemplarze) otrzymano przy odżywianiu *Glyceria fluitans*, nieco mniejsze (śr. 4 166) przy odżywianiu *Poa pratensis*, 3 655 przy odżywianiu *Alopecurus pratensis*, najmniej 516 — 313 przy odżywianiu *Aira caespitosa*. Przy odżywianiu wymoczków liśćmi koniczyny obserwuje się stały spadek (od pierwszego dnia hodowli) ilości wymoczków, które w pięciu równolegle prowadzonych kulturach wyginęły od 13 do 21 dnia hodowli (Tabela V). Ilość wymoczków jaką otrzymano w różnych kulturach jest w dużym stopniu wskaźnikiem świadczącym o wartości liści traw jako pokarmu dla *A. denticulatum* będącego ogniwem wiążącym przeżuwacza z fauną wymoczkową żołądka w przemianie białek roślinnych.